EXHIBIT A

EXHIBIT A:
AMENDMENTS MADE TO PENDING CLAIMS
U.S. PATENT APPLICATION SERIAL NO. 09/493,353
(ATTORNEY DOCKET NO. 2094/1E286-US1)

SUBMITTED PURSUANT TO 37 C.F.R. § 1.121(c)(1)(ii)

1. (Amended) A method for detecting the presence of Hepatitis C Virus (HCV) RNA in a biological sample, said method comprising:

(A) performing a reverse transcription reaction using, as a template, RNA derived from said sample to produce HCV-specific reverse transcription products;

(B) amplifying said reverse-transcription products using one or more pairs of oligonucleotide primers specific for HCV to produce HCV-specific amplification products,

wherein said pairs are selected from the group consisting of:

(a) forward primer 5'-CAGAAAGCGTCTAGCCATGGCGTTAGTA-3' (C69F28) <SEQ ID NO. 1> and reverse primer 5'-CGGTTCCGCAGAGACCACTATGGCTCTC-3' (C133R26) <SEQ ID NO. 4>; [or]

(b) forward primer 5'-GGGAGAGCCATAGTGGTCTGCGGAA-3' (C131F25) <SEQ ID NO. 2> and reverse primer 5'-CGGGGCACTCGCAAGCACCCTATCA-3' (C294R25) <SEQ ID NO. 7>; and

(c) forward primer 5'-GTGGTCTGCGGAACCGGTGAGTACAC-[3] 3' (C143F26) <SEQ ID NO. 3> and a reverse primer selected from the group consisting of

(i) 5'-GCAAGCACCCTATCAGGCAGTACCACA-3' (C282R27) <SEQ ID NO. 5>,

(ii) 5'-CACTCGCAAGCACCCTATCAGGCAGTA-3' (C287R27) <SEQ ID NO. 6>; and

(C) detecting said amplification products,

wherein detection of said amplification products indicates the presence of HCV RNA in said sample.

9. (Amended) A method for amplifying Hepatitis C Virus (HCV) DNA, [said] which method comprises [comprising:

(A)] performing a polymerase chain reaction on a DNA sample containing HCV DNA using one or more pairs of oligonucleotide primers specific for HCV to produce HCV-specific amplification products,

wherein said pairs are selected from the group consisting of:

(a) forward primer 5'-CAGAAAGCGTCTAGCCATGGCGTTAGTA-3' (C69F28) <SEQ ID NO. 1> and reverse primer

5'-CGGTTCCGCAGACCACTATGGCTCTC-3' (C133R26) <SEQ ID NO. 4>; [or]

(b) forward primer 5'-GGGAGAGCCATAGTGGTCTGCGGAA-3' (C131F25) <SEQ ID NO. 2> and reverse primer 5'-CGGGGCACTCGCAAGCACCCTATCA-3' (C294R25) <SEQ ID NO. 7>; and

(c) forward primer 5'-GTGGTCTGCGGAACCGGTGAGTACAC-3 (C143F26) <SEQ ID NO. 3> and a reverse primer selected from the group consisting of

(i) 5'-GCAAGCACCCTATCAGGCAGTACCACA-3' (C282R27) <SEQ ID NO. 5>,

(ii) 5'-CACTCGCAAGCACCCTATCAGGCAGTA-3' (C287R27) <SEQ ID NO. 6>.

10. (Amended) A method as defined in claim 9, which method further comprises [comprising:

(B)] detecting said amplification products,

wherein detection of said amplification products indicates the presence of HCV DNA in said sample.

22. (Amended) A method for amplifying Hepatitis C Virus (HCV) DNA, [said] which method comprises [comprising:

(A)] performing a polymerase chain reaction on a DNA sample containing HCV DNA using a forward primer and a reverse primer to produce HCV-specific amplification products,

wherein said forward primer consists of the oligonucleotide 5'-GGTGGCTCCATCTTAGCCCTAGTCACG- 3' (1F27) <SEQ ID NO. 8> and said reverse primer consists of the oligonucleotide 5'-AGGCCAGTATCAGCACTCTCTGCAGTC-3' (57R27) <SEQ ID NO. 9>.

23. (Amended) A method as defined in claim 22, which method further comprises [comprising:

(B)] detecting said amplification products,

wherein detection of said amplification products indicates the presence of HCV DNA in said sample.

27. (Amended) A method for detecting the presence of Hepatitis C Virus (HCV) RNA in a biological sample, said method comprising:

(A) performing a reverse transcription reaction using as a template RNA derived from said sample to produce HCV-specific reverse transcription products;

(B) amplifying said reverse-transcription products using one or more pairs of 5' NCR oligonucleotide primers specific for HCV and one or more pairs of 3' NCR oligonucleotide primers to produce HCV-specific amplification products,

wherein said 5' NCR primer pairs are selected from the group consisting of:

(a) forward primer 5'-CAGAAAGCGTCTAGCCATGGCGTTAGTA-3' (C69F28) <SEQ ID NO. 1> and reverse primer 5'-CGGTTCCGCAGACCACTATGGCTCTC-3' (C133R26) <SEQ ID NO. 4>; [or]

(b) forward primer 5'-GGGAGAGCCATAGTGGTCTGCGGAA-3' (C131F25) <SEQ ID NO. 2> and reverse primer 5'-CGGGGCACTCGCAAGCACCCTATCA-3' (C294R25) <SEQ ID NO. 7>; and

(c) forward primer 5'-GTGGTCTGCGGAACCGGTGAGTACAC-3' (C143F26) <SEQ ID NO.3> and a reverse primer selected from the group consisting of

(i) 5'-GCAAGCACCCTATCAGGCAGTACCACA-3' (C282R27) <SEQ ID NO. 5>,

(ii) 5'-CACTCGCAAGCACCCTATCAGGCAGTA-3' (C287R27) <SEQ ID NO. 6>; and

wherein each of said pairs of 3' NCR oligonucleotide primers comprises a forward primer consisting of the oligonucleotide 5'-GGTGGCTCCATCTTAGCCCTAGTCACG-3' (1F27) <SEQ ID NO. 8> and a reverse primer consisting of the oligonucleotide 5'-AGGCCAGTATCAGCACTCTCTGCAGTC-[3] 3' (57R27) <SEQ ID NO. 9>; and

(C) detecting said amplification products,

wherein detection of said amplification products indicates the presence of HCV RNA in said sample.

35. (Amended) A method for amplifying Hepatitis C Virus (HCV) DNA, [said] which method comprises [comprising:

(A)] performing a polymerase chain reaction on a DNA sample containing HCV DNA using one or more pairs of 5' NCR oligonucleotide primers specific for HCV and one or more pairs of 3' NCR oligonucleotide primers to produce HCV-specific amplification products,

wherein said 5' NCR primer pairs are selected from the group consisting of:

(a) forward primer 5'-CAGAAAGCGTCTAGCCATGGCGTTAGTA-3' (C69F28) <SEQ ID NO. 1> and reverse primer

5'-CGGTTCCGCAGACCACTATGGCTCTC-3' (C133R26) <SEQ ID NO. 4>; [or]

(b) forward primer 5'-GGGAGAGCCATAGTGGTCTGCGGAA-3' (C131F25) <SEQ ID NO. 2> and reverse primer 5'-CGGGGCACTCGCAAGCACCCTATCA-3' (C294R25) <SEQ ID NO. 7>; and

(c) forward primer 5'-GTGGTCTGCGGAACCGGTGAGTACAC-3' (C143F26) <SEQ ID NO. 3> and a reverse primer selected from the group consisting of

(i) 5'-GCAAGCACCCTATCAGGCAGTACCACA-3' (C282R27) <SEQ ID NO. 5>,

(ii) 5'-CACTCGCAAGCACCCTATCAGGCAGTA-3' (C287R27) <SEQ ID NO. 6>; and

wherein each of said pairs of 3' NCR oligonucleotide primers comprises a forward primer consisting of the oligonucleotide 5'-GGTGGCTCCATCTTAGCCCTAGTCACG-3' (1F27) <SEQ ID NO. 8> and a reverse primer consisting of the oligonucleotide 5'-AGGCCAGTATCAGCACTCTCTGCAGTC-[3] 3' (57R27) <SEQ ID NO. 9>.

36. (Amended) A method as defined in claim 35, which method further comprises [comprising:

(B)] detecting said amplification products,

wherein detection of said amplification products indicates the presence of HCV DNA in said sample.

40. (Amended) An oligonucleotide selected from the group consisting of:

5'-CAGAAAGCGTCTAGCCATGGCGTTAGTA-3' (C69f28) <SEQ ID NO. 1>[.];

5'-GGGAGAGCCATAGTGGTCTGCGGAA-3' (C131F25) <SEQ ID NO. 2>[.];

5'-GTGGTCTGCGGAACCGGTGAGTACAC-3 (C143F26) <SEQ ID NO. 3>[.];

5'-CGGTTCCGCAGACCACTATGGCTCTC-3 (C133R26) <SEQ ID NO. 4>[.];

[5'-GCAAGCACCCTATCAGGCAGTACCACA-3' (C282R27) <SEQ ID NO. 5>.]

5'-CACTCGCAAGCACCCTATCAGGCAGTA-3' (C287R27) <SEQ ID NO. 6>[.];

5'-CGGGGCACTCGCAAGCACCCTATCA-3' (C294R25) <SEQ ID NO. 7)[.];

5'-GGTGGCTCCATCTTAGCCCTAGTCACG-3' (1F27) <SEQ ID NO. 8>[.];

5'-AGGCCAGTATCAGCACTCTCTGCAGTC-3 (57R27) <SEQ ID NO. 9>[.];

5'-GGGTCCTGGAGGCTGCACGACACTCAT-3' (C96-22-PRB) <SEQ ID NO. 11>[.];

5'-CCTTTCGCGACCCAACACTACTCGGCT-3' (C252-27-PRB) <SEQ ID NO. 12>[.];

5'-TTTCGCGACCCAACACTACTCGGCT-3' (C252-25-PRB) <SEQ ID NO. 13>[.];

5'-GCGGCTCACGGACCTTTCACAGCTA-3' (30PRB25) <SEQ ID NO. 14>[.];

and

5'-ATGCGGCTCACGGACCTTTCACAGC-3' (32PRB25) <SEQ ID NO. 15>.

41. (Amended) An HCV-specific amplification primer oligonucleotide selected from the group consisting of:

5'-CAGAAAGCGTCTAGCCATGGCGTTAGTA-3' (C69F28) <SEQ ID NO. 1>[.];

5'-GGGAGAGCCATAGTGGTCTGCGGAA-3' (C131F25) <SEQ ID NO. 2>[.];

5'-GTGGTCTGCGGAACCGGTGAGTACAC-3' (C143F26) <SEQ ID NO. 3>[.];

5'-CGGTTCCGCAGACCACTATGGCTCTC-3' (C133R26) <SEQ ID NO. 4>[.];

[5'-GCAAGCACCCTATCAGGCAGTACCACA-3' (C282R27) <SEQ ID NO. 5>.]

5'-CACTCGCAAGCACCCTATCAGGCAGTA-3' (C287R27) <SEQ ID NO. 6>[.];

5'-CGGGGCACTCGCAAGCACCCTATCA-3' (C294R25) <SEQ ID NO. 7>[.];

5'-GGTGGCTCCATCTTAGCCCTAGTCACG-3' (1F27) <SEQ ID NO. 8>[.]; and

5'-AGGCCAGTATCAGCACTCTCTGCAGTC-3' (57R27) <SEQ ID NO. 9>.

42. (Amended) [A] An oligonucleotide probe comprising [an oligonucleotide] a sequence selected from the group consisting of:

5'-GGGTCCTGGAGGCTGCACGACACTCAT-3' (C96-22-PRB) <SEQ ID NO. 11>[.];

5'-CCTTTCGCGACCCAACACTACTCGGCT-3' (C252-27-PRB) <SEQ ID NO. 12>[.];

5'-TTTCGCGACCCAACACTACTCGGCT-3' (C252-25-PRB) <SEQ ID NO. 13>[.];

5'-GCGGCTCACGGACCTTTCACAGCTA-3' (30PRB25) <SEQ ID NO. 14>[.]; and

5'-ATGCGGCTCACGGACCTTTCACAGC-3' (32PRB25) <SEQ ID NO. 15>.

54. (Amended) A kit for detecting the presence of HCV DNA, said kit comprising one or more pairs of 5' NCR oligonucleotide primers, wherein said 5' NCR primer pairs are selected from the group consisting of:

(a) forward primer 5'-CAGAAAGCGTCTAGCCATGGCGTTAGTA-3' (C69F28) <SEQ ID NO. 1> and reverse primer 5'-CGGTTCCGCAGACCACTATGGCTCTC-3' (C133R26) <SEQ ID NO. 4>; [or]

(b) forward primer 5'-GGGAGAGCCATAGTGGTCTGCGGAA-3' (C131F25) <SEQ ID NO. 2> and reverse primer 5'-CGGGGCACTCGCAAGCACCCTATCA-3' (C294R25) <SEQ ID NO. 7>; and

(c) forward primer 5'-GTGGTCTGCGGAACCGGTGAGTACAC-[3] 3' (C143F26) <SEQ ID NO. 3> and a reverse primer selected from the group consisting of

(i) 5'-GCAAGCACCCTATCAGGCAGTACCACA-3' (C282R27)

<SEQ ID NO. 5>,

(ii) 5'-CACTCGCAAGCACCCTATCAGGCAGTA-3' (C287R27)

<SEQ ID NO. 6>.

EXHIBIT B

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Preclinical Evaluation of AMPLICOR Hepatitis C Virus Test for Detection of Hepatitis C Virus RNA

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We compared a single-enzyme, combined reverse transcription-PCR (RT-PCR; AMPLICOR HCV Test; Roche Molecular Systems, Branchburg, N.J.) with an independent, two-enzyme, standard RT-PCR (SRT-PCR) assay for the detection of hepatitis C virus (HCV) RNA in serum and plasma. Test samples included a proficiency testing panel consisting of 10 undiluted plasma samples, three separate dilution series, and sera from 99 patients with chronic liver disease. The quantity of HCV RNA in each patient serum sample was determined by a branched DNA (bDNA) signal amplification assay (Quantiplex HCV-RNA assay; Chiron, Emeryville, Calif.). There was complete concordance between the results of the RT-PCR assays with the 10 undiluted plasma samples used for proficiency testing (3 positive and 7 negative samples). However, the analytical sensitivity of SRT-PCR was 4- to 10-fold greater than that of the AMPLICOR test in the dilution series. HCV RNA was detected in 44, 45, and 40 of the patient serum samples, by SRT-PCR, the AMPLICOR test, and the bDNA assay, respectively. There was 97% agreement between the results of the RT-PCR assays, with only three discrepancies. Review of the patients' medical records resolved all three discrepancies in favor of the AMPLICOR results (two false-negative SRT-PCR results and one false-positive SRT-PCR result). The quantity of HCV RNA in sera from five (11%) patients with viremia detected by AMPLICOR was below the bDNA assay cutoff (<3.5 × 10^5 RNA equivalents per ml). AMPLICOR compared favorably with SRT-PCR, with key advantages of speed, ease of use, increased sample throughput, and protection against false-positive results because of amplicon carryover.

Hepatitis C virus (HCV) is the principal cause of posttransfusion and sporadic non-A, non-B hepatitis in the world (3). HCV is a single-stranded, positive-sense RNA virus with a genome of approximately 10,000 nucleotides coding for 3,000 amino acids (9). The development of immunoserological tests for anti-HCV antibodies has facilitated the detection of HCV infection and has reduced the incidence of posttransfusion hepatitis in the United States (10).

Unfortunately, these immunoserological tests have several shortcomings. Anti-HCV antibody tests may have a prolonged window of seronegativity after acute infection, do not differentiate between active and resolved infections, and may be falsely positive in patients with hypergammaglobulinemia and other forms of chronic hepatitis such as autoimmune hepatitis (1, 5, 6). Falsely negative antibody tests may occur in as many as 10% of patients with chronic HCV infection (1). Currently, an immunological assay for the direct detection of HCV antigen in serum is not available.

Amplification of viral cDNA by reverse transcription-PCR (RT-PCR) has been shown to be a sensitive means for the direct detection of HCV (7, 8, 20). Detection of HCV RNA in serum is indicated for patients with acute hepatitis prior to seroconversion, in suspected cases of chronic HCV infection in seronegative patients, for the resolution of indeterminate immunoserological tests, and to monitor patients receiving alpha interferon therapy (9, 11, 15).

Since the genome of HCV is a single-stranded RNA molecule, PCR amplification must be preceded by a step that generates a cDNA copy. Synthesis of the cDNA is usually accomplished by using a retroviral reverse transcriptase. Amplification of the cDNA is typically done with a thermostable DNA polymerase from *Thermus aquaticus*. The need for different enzymes in the two steps initially led to the addition of new enzyme and a change of buffer conditions between the RT and DNA amplification steps. The two-step process is cumbersome and increases the opportunity for contamination of the reaction mixture with previously amplified DNA. Coupled RT-PCR assays in which retroviral reverse transcriptase, *Taq* polymerase, and both primers are present in a single buffer system have also been described (4).

A combined RT-PCR assay for HCV RNA has been developed by Roche Molecular Systems (AMPLICOR HCV test). The assay uses a single thermostable enzyme and buffer condition in a single reaction tube (21). The DNA polymerase of *Thermus thermophilus* (*rTth*) is used in the AMPLICOR test because of its enhanced RT activity in the presence of manganese (14). In addition to minimizing the possibility of contamination, the use of a thermostable enzyme in the AMPLICOR test allows for RT at elevated temperatures. This should increase the specificity of primer extension and improve the efficiency of RT through destabilization of the secondary RNA structure. In addition, the use of a thermostable enzyme for RT is compatible with the uracil-*N*-glycosylase (UNG) protocol for prevention of false-positive results because of the carryover of previously amplified DNA (13).

The branched DNA (bDNA) assay is a quantitative signal amplification method based on a series of specific hybridization reactions and chemiluminescent detection of hybridized probes in a microwell format (18). Quantitative determination of HCV RNA in serum may provide important prognostic information and a marker of response to interferon therapy (11, 12). However, the high threshold of detection of the bDNA assay (3.5 × 10^5 HCV RNA equivalents per ml) may limit its use in the diagnosis and management of patients with HCV infection.

In the study described here we compared the AMPLICOR HCV test with an independent, two-enzyme, standard RT-PCR assay (SRT-PCR) for the detection of HCV RNA in serum and plasma. Test samples included a proficiency testing

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panel, three separate dilution series, and sera from 99 patients with chronic liver disease. The quantity of HCV RNA in each patient serum sample was also determined by the bDNA assay (Quantiplex HCV RNA assay; Chiron, Emeryville, Calif.).

MATERIALS AND METHODS

Clinical specimens. The sera included in the present evaluation were obtained from 99 patients referred to a university hepatology clinic who were undergoing evaluation for chronic liver disease. Frozen sera from our serum bank were selected for this evaluation on the basis of SRT-PCR results (44 serum samples were positive and 55 serum samples were negative for HCV RNA). All serum samples were separated from the clots within 4 h of collection, divided into aliquots, and stored at −70°C until they were tested by the AMPLICOR test and the bDNA assay. A serial 10-fold dilution series of serum from a patient (patient 844) containing 39.3×10^5 HCV RNA equivalents per ml by the bDNA assay was prepared in HCV-negative serum to compare the limits of detection of the RT-PCR-based assays.

Patients. The age, sex, diagnosis, liver histology, risk factors for HCV infection, liver function test results (alanine aminotransferase, asparatate aminotransferase, alkaline phosphatase, total bilirubin, and serum albumin), and HCV serological status of each patient were obtained by review of the patients' medical records. Forty-three (44%) patients were diagnosed as having chronic HCV infection, while the remainder were considered to have a variety of other liver diseases; cryptogenic cirrhosis, 12%; alcoholic liver disease, 10%; autoimmune hepatitis, 7%; primary biliary cirrhosis, 6%; polycystic liver disease, 3%; chronic hepatitis B virus infection, 2%; other chronic liver diseases, 10%; unspecified, 5%. The results of HCV antibody testing (enzyme immunoassay [EIA] EIA-1 or EIA-2, Abbott Laboratories, Abbott Park, Ill.) were available for 95 patients, and 45 (47%) had positive test results.

Eurohep proficiency testing panel. The second Eurohep HCV RNA proficiency testing panel was tested by both the AMPLICOR test and SRT-PCR (21). The coded test panel consisted of 10 undiluted plasma samples and two separate dilution series. The Eurohep dilution series 1 and 2 contained dilutions of HCV genotypes 1 and 3, respectively, in negative plasma.

SRT-PCR. The primer pair used in the SRT-PCR assay was selected from the highly conserved 5′-untranslated region of the HCV genome. The pair consisted of downstream primer 5PUT c1-a, 5′-CCCAACACTACTCGCCTAG-3′ (nucleotides −74 to −92), and upstream primer 5PUT 1-s, 5′-AACTACTGTCTTCACGCAG AAAGC-3′ (nucleotides −266 to −289) (2). 5PUT p1-s, 5′-GCCATGGCGTTAG TATGAGTGTC-3′ (nucleotides −238 to −260), served as a hybridization probe.

Each serum sample was thawed and divided into equal 100-μl aliquots. RNA was extracted in duplicate with RNAzol B (Biotecx Laboratories, Houston, Tex.) as described previously (17). RNA pellets were dissolved in 10 μl of RNase-free 1 mM EDTA–10 mM NaCl–10 mM Tris-HCl (pH 8.0). One-half volume (5 μl) of the dissolved RNA pellet was added to 15 μl of the RT reaction mixture (GeneAmp RNA PCR kit; Perkin-Elmer Cetus, Norwalk, Conn.) consisting of 5 mM $MgCl_2$, 1× Buffer II (Perkin-Elmer Cetus), 1 mM (each) deoxyribonucleoside triphosphate (dATP, dCTP, dGTP, and dTTP), 1 U of RNase inhibitor, 2.5 U of Moloney leukemia virus reverse transcriptase, and 0.75 μM downstream primer (5PUT c1-a). cDNA synthesis was performed under mineral oil in a GeneAmp 480 thermocycler (Perkin-Elmer Cetus) with one cycle at 42°C for 15 min, one cycle at 99°C for 5 min, and one cycle at 4°C for 5 min.

A total of 80 μl of the PCR mixture consisting of 2 mM $MgCl_2$, 1× Buffer II, 2.5 U of Amplitaq, and 0.15 μM upstream primer (5PUT 1-s) was added to each tube under the oil layer. PCR amplification proceeded with incubation at 94°C for 5 min; this was followed by 5 cycles of 94°C for 2 min, 50°C for 2 min, and 72°C for 3 min and then 30 cycles of 94°C for 1.5 min, 60°C for 2 min, and 72°C for 3 min and a final extension step at 72°C for 7 min.

After completion of the amplification reaction, 25 μl of each reaction mixture was analyzed by electrophoresis through a 2.0% agarose gel with ethidium bromide staining and Southern blotting. DNA was transferred onto Duralon (Statagene, La Jolla, Calif.) nylon membranes by alkaline transfer (19). The transferred DNA was cross-linked to the membrane by UV light (Stratalinker; Stratagene). The blots were prehybridized in a solution of 0.05 M sodium phosphate buffer (pH 7.2), 1 mM EDTA, 7% sodium dodecyl sulfate (SDS), and 1% bovine serum albumin, at 42°C for 15 min in a shaking water bath. The prehybridization buffer was removed and the blots were hybridized in the same buffer with a biotin 3′-end-labeled probe (Clontech, Palo Alto, Calif.), 5PUT p1-s, at 42°C for 1 h. The blots were washed for 20 min at 55°C in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) with 0.1% SDS. A chemiluminescent Southern blot procedure was used to detect the hybridized probe (Southern-Light; Tropix, Bedford, Mass.). Results were imaged on X-ray film after 30 to 60 min of exposure. If the results of duplicate tests did not agree, the samples were reextracted and reamplified in duplicate.

A negative control serum sample, a reagent blank, and a positive control serum sample containing approximately 1,000 copies of the HCV genome per ml were included with each batch of specimens. The positive and negative control sera were carried through all of the steps in the procedure from extraction to product detection. The recommended procedures were followed to prevent false-positive reactions as a result of target or amplified product contamination (16).

AMPLICOR HCV test. HCV RNA was isolated from serum by guanidinium thiocyanate lysis and isopropanol precipitation in the presence of poly(A) carrier RNA. An equivalent of 5 μl of serum was amplified in a master mixture that contained *rTth* DNA polymerase, the primers KY80 and KY78, buffer salts, UNG, dATP, dCTP, dGTP, and dUTP. dUTP is incorporated into each amplification product to serve as a substrate for UNG (AmpErase; Roche Molecular Systems) to prevent carryover contamination of previously amplified DNA. A single primer pair, including a biotinylated downstream primer (KY80, 5′-GCAGAAAGCGTCT AGCCATGGCGT, and KY78, 5′-biotinyl-CTCGCAAGCACCCTATCAGGCA GT) was used to define a 244-bp amplicon located in the 5′-untranslated region of the HCV genome. The reaction was optimized for the use of *rTth* that, in the presence of manganese, performs both RT and DNA polymerase functions, obviating the requirement for two enzymes and two separate reactions. Amplification was carried out in the GeneAmp 9600 thermocycler (Perkin-Elmer Cetus) with a program that allowed for a 2-min incubation at 50°C for optimal UNG activity; this was followed by 30 min of incubation at 60°C for the reverse transcriptase step, 40 cycles of PCR (2 cycles of 95°C for 15 s and 60°C for 20 s and 38 cycles of 90°C for 15 s and 60°C for 20 s), and a final 4-min extension at 60°C. Detection of the PCR product was accomplished through the use of a solid-phase probe, specific for HCV, that was coated onto microwell plates. The biotin-labeled PCR product was chemically denatured to form single strands, hybridized to the microwells, and detected by using an avidin-horseradish peroxidase system with a conventional microtiter plate washer and a microtiter plate reader (450 nm). Optical density readings of >0.500 were considered positive, those of <0.300 were considered negative, and those of 0.300 to 0.500 were considered equivocal.

All samples were extracted once, and a single amplification and detection were performed on each extract. One positive and three negative controls provided with the kit were run with each batch of patient specimens. The controls were not extracted with the lysis buffer. By these methods, the time required to obtain a result is approximately 6 h for a batch size of 25 samples.

bDNA assay. Quantitative bDNA signal amplification assays (Quantiplex HCV assay; Chiron) were performed according to the manufacturer's instructions as described previously (17). The limit of detection was 3.5×10^5 HCV RNA equivalents per ml. A positive and a negative control serum sample as well as the four calibration standards were run with each bDNA assay.

RESULTS

There was complete concordance between the results of the SRT-PCR and the AMPLICOR assays for the 10 undiluted plasma samples that were part of the Eurohep proficiency testing panel (3 positive and 7 negative serum samples). However, testing of three separate dilution series (Eurohep-1, Eurohep-2, and patient 844) by both RT-PCR assays showed that SRT-PCR had a greater analytical sensitivity, with a ≥4- to 10-fold difference in the limit of detection of HCV RNA between the two assays in these terminal dilution experiments. The last dilution positive by the AMPLICOR test in the dilution series prepared from patient 844 contained approximately 400 HCV RNA equivalents per ml.

HCV RNA was detected in 44 and 45 serum samples by SRT-PCR and the AMPLICOR test, respectively. No equivocal results were obtained by the AMPLICOR test. Overall, there was a 97% concordance between the results of the two RT-PCR assays, with only three discordant results. A total of 43 serum samples were positive by both SRT-PCR and the AMPLICOR test, 53 serum samples were negative by both tests, 2 serum samples were positive by the AMPLICOR test but negative by SRT-PCR, and 1 serum sample was negative by the AMPLICOR test and positive by SRT-PCR.

The resolution of the discordant results is summarized in Table 1. Patient 104 (SRT-PCR negative, AMPLICOR positive) was a 41-year-old male with elevated transaminase levels in his serum and chronic active hepatitis and cirrhosis on liver biopsy. This patient had no identifiable risk factors for HCV infection. Anti-HCV EIA and supplementary recombinant immunoblot assay (RIBA) were both positive, and other causes of liver disease were excluded by serological testing. Patient 910 (SRT-PCR negative, AMPLICOR positive) was a 37-year-old male originally thought to have cirrhosis secondary to alcohol abuse. This patient also had a history of blood transfusions and intravenous drug abuse. Anti-HCV EIA and supplementary RIBA were positive, and other causes of liver disease were excluded by serological testing. Patient 1114 (SRT-PCR positive, AMPLICOR negative) was a 57-year-old female with cryptogenic cirrhosis. She had no identifiable risk factors for HCV infection. All serological tests including anti-HCV EIA and RIBA were negative. Two additional serum samples, collected at different times, were tested for HCV RNA by SRT-

TABLE 1. Resolution of samples with discordant results by SRT-PCR and the AMPLICOR test

Patient no.[a]	EIA result	RIBA result	ALT level (U/liter)[b]	Risk factor	AMPLICOR test result	SRT-PCR result	Interpretation
104	+	+	90	None	+	−	Low-level viremia
910	+	+	73	Transfusion, IVDA[c]	+	−	Low-level viremia
1114	−	−	46	None	−	+[d]	False-positive SRT-PCR result

[a] The bDNA assay result was $<3.5 \times 10^5$ HCV RNA equivalents per ml for all patients. Histology for all patients was cirrhosis.
[b] ALT, alanine aminotransferase.
[c] IVDA, intravenous drug abuse.
[d] Date of positive SRT-PCR result was 21 July 1993; SRT-PCR assays done on 3 June and 27 September 1993 were negative.

PCR and were found to be negative. After review of the patients' charts, all three discrepant results were resolved in favor of the AMPLICOR test (two false-negative SRT-PCR results and one false-positive SRT-PCR result). The levels of viremia in both serum samples with false-negative SRT-PCR results were $<3.5 \times 10^5$ HCV RNA equivalents per ml by the bDNA assay.

Figure 1 shows the frequency distribution of the quantity of HCV RNA in the test sera which were positive by the AMPLICOR test. HCV viremia was documented by the AMPLICOR test in all sera with $\geq 3.5 \times 10^5$ HCV RNA equivalents per ml of serum and in five (11%) serum samples that contained RNA at a quantity that was below the threshold of detection by the bDNA assay. No serum sample was positive by the bDNA assay alone. The level of viremia was $>1 \times 10^6$ HCV RNA equivalents per ml in 31% of viremic patients, $>10 \times 10^6$ HCV RNA equivalents per ml in 24% of viremic patients, and $>57 \times 10^6$ HCV RNA equivalents per ml in 7% of viremic patients.

The HCV genome was detected by the AMPLICOR test in three (6%) of the anti-HCV-negative specimens. All three specimens were also positive for HCV RNA by SRT-PCR and the bDNA assay. HCV RNA was not detected in six (13%) of the anti-HCV-positive specimens by the AMPLICOR test, SRT-PCR, or the bDNA assay. For patients with a clinical diagnosis of chronic HCV infection, 89% were positive for HCV RNA by AMPLICOR and 90% were positive for anti-HCV antibodies.

DISCUSSION

The results of a recent HCV RNA proficiency testing survey illustrated the need for increased standardization (22). In that survey, one-third of the laboratories had errors in determining the status of undiluted samples, and one-half had one or more errors in the dilution series. Only 16% of participating laboratories had no errors, and even in those laboratories there was a 100-fold difference in analytical sensitivity. The development of a reliable kit assay should increase the availability, standardization, and reliability of HCV RNA detection. To date, there are no HCV RNA assays licensed by the U.S. Food and Drug Administration for diagnostic use.

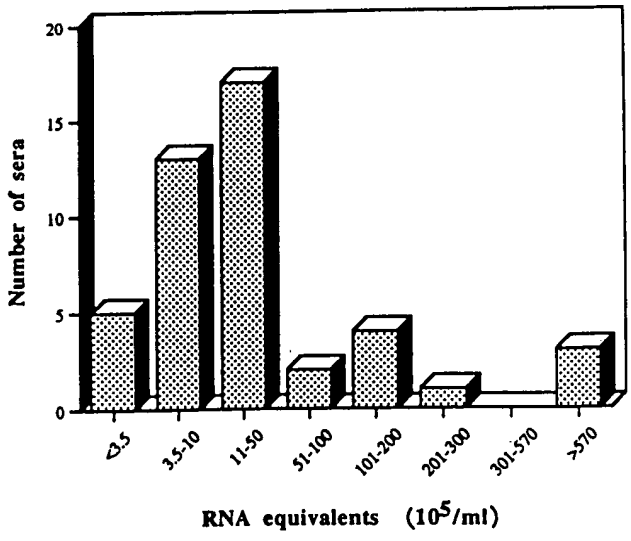

FIG. 1. Frequency distribution of the quantity of HCV RNA in 45 test serum samples which were positive by AMPLICOR.

We compared the AMPLICOR HCV test with an SRT-PCR assay for the detection of HCV RNA in a plasma proficiency testing panel and in sera from patients with chronic liver disease. The SRT-PCR assay reliably detected between 10 and 100 copies of the HCV genome per ml of serum (data not shown). We found that the SRT-PCR assay had a >4- to 10-fold higher analytical sensitivity than the AMPLICOR test in the three dilution series tested. The difference in sensitivity may be explained on the basis of the amount of RNA put into each reaction tube. RNA isolated from the equivalent of 5 μl of serum is amplified in each AMPLICOR test, whereas RNA isolated from the equivalent of 50 μl of serum is amplified in each SRT-PCR assay. By using the bDNA assay to quantitate the HCV RNA in a patient serum sample, we estimated the analytical sensitivity of the AMPLICOR test to be approximately 400 HCV genome equivalents per ml.

When the results of both RT-PCR assays were compared for the undiluted patient sera, we found 97% agreement. Despite the apparent lower analytical sensitivity of the AMPLICOR test, it detected HCV RNA in two serum samples that were negative in the SRT-PCR assay. Both patients had serological and clinical evidence of HCV infection and low-level viremia ($<3.5 \times 10^5$ HCV RNA equivalents per ml). The false-negative SRT-PCR results may have been due to inhibition of the amplification reaction. The SRT-PCR may be more prone to inhibition than the AMPLICOR test since the sample input in each reaction is larger. The inhibitors could be endogenous and patient specific or could be related to carryover of the chaotrope and organic solvents used in the RNA extraction procedure. Neither RT-PCR assay included an internal control template that could be used to assess the efficacy of each negative amplification reaction.

Zeuzem and colleagues (23) compared the AMPLICOR HCV test with a two-step, single-primer-pair RT-PCR for the detection of HCV RNA in sera obtained from 219 consecutive patients attending a hepatology clinic. Dilution of HCV RNA transcripts into control serum showed an analytical sensitivity of 5×10^3 to 1×10^4 molecules per ml of serum for the AMPLICOR test and 1×10^3 molecules per ml for the two-step RT-PCR. Despite the lower analytical sensitivity of the AMPLICOR test, no false-negative results were obtained with the AMPLICOR test with sera from 111 viremic patients (100% sensitivity). The magnitude of viremia in these patients was estimated to range from 5×10^5 to 5×10^8 genome equivalents per ml. There were three false-positive AMPLICOR test results with sera from 108 control patients (97% specificity).

The positive and negative controls for the AMPLICOR test were not subjected to the RNA extraction steps. Ideally, a positive control should contain the target nucleic acid in a matrix similar to that of the clinical samples to be tested and should be subjected to all of the steps of the assay procedure to ensure efficient sample preparation. Likewise, the negative control should be similar to the clinical samples tested and should be subjected to all of the steps to ensure that contamination did not occur.

The AMPLICOR test has two features that should limit false-positive results because of carryover of amplified product from one reaction mixture to another. It is a single-enzyme, single-tube reaction to which no reagents are added between the RT and the amplification steps. This eliminates the need to open the tube after the initial setup and reduces the possibility of contamination. Also, the reaction mixture includes UNG, which recognizes and catalyzes the destruction of dU-containing DNA. The presence of dU in amplified product renders any contaminating product susceptible to destruction by UNG prior to amplification of the target cDNA. We found one false-positive SRT-PCR result and no false-positive AMPLICOR test results in the present study.

The enzyme-linked immunosorbent assay (ELISA)-like detection format of the AMPLICOR test can potentially be another source of false-positive results (23). Unbound horseradish peroxidase could reenter the wells after washing if the wells were overfilled, and the contamination could lead to significant color development. Smaller wash volumes could reduce the occurrence of false-positive results by the detection system (23). False-positive results by the detection system were not encountered in the present study.

We tested all patient serum samples by the bDNA assay to provide a third independent RNA detection method and to determine the levels of viremia in our patient population. The magnitude of viremia was $>3.5 \times 10^5$ HCV RNA equivalents per ml in 89% of the patients in whose sera HCV RNA was detected by RT-PCR. The performance characteristics of the AMPLICOR test need to be assessed with sera from other patient groups with lower levels of viremia, especially patients receiving interferon therapy.

The format of the AMPLICOR test is better suited than the SRT-PCR assay to a clinical laboratory setting. The AMPLICOR test is less labor intensive and can be completed in approximately 6 h, whereas 48 h is required for completion of the SRT-PCR. The use of a single enzyme and buffer system for RT and cDNA amplification simplifies the RT-PCR process and reduces the opportunity for contamination. The AMPLICOR test uses familiar "ELISA" technology rather than cumbersome gel analysis and Southern blotting for the detection of amplified product.

The major limitations of the AMPLICOR test include the relatively small sample volume, the lack of an internal positive control, and failure to provide controls for the RNA extraction step. The AMPLICOR test used in the present study does not represent a final product, and it will be modified prior to large-scale clinical trials in the United States. The modifications will include a larger effective sample volume and kit controls that are carried through the RNA extraction step. We are evaluating the modified protocol.

In conclusion, the AMPLICOR test compared favorably with our SRT-PCR for the detection of HCV RNA in sera from patients with chronic liver disease in the present preclinical evaluation. The commercial development of a reliable kit assay should increase the availability and improve the standardization of testing.

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EXHIBIT C

CURRENT PROTOCOLS IN MOLECULAR BIOLOGY

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VOLUME 4

THE POLYMERASE CHAIN REACTION

15

continued

Enzymatic Amplification of DNA by PCR: Standard Procedures and Optimization

BASIC PROTOCOL

This unit describes a method for amplifying DNA enzymatically by the polymerase chain reaction (PCR), including procedures to quickly determine conditions for successful amplification of the sequence and primer sets of interest, and to optimize for specificity, sensitivity, and yield. The first step of PCR simply entails mixing template DNA, two appropriate oligonucleotide primers, *Taq* or other thermostable DNA polymerases, deoxyribonucleoside triphosphates (dNTPs), and a buffer. Once assembled, the mixture is cycled many times (usually 30) through temperatures that permit denaturation, annealing, and synthesis to exponentially amplify a product of specific size and sequence. The PCR products are then displayed on an appropriate gel and examined for yield and specificity.

Many important variables can influence the outcome of PCR. Careful titration of the $MgCl_2$ concentration is critical. Additives that promote polymerase stability and processivity or increase hybridization stringency, and strategies that reduce nonspecific primer-template interactions, especially prior to the critical first cycle, generally improve amplification efficiency. This protocol, using *Taq* DNA polymerase, is designed to optimize the reaction components and conditions in one or two stages. The first stage (steps 1 to 7) determines the optimal $MgCl_2$ concentration and screens several enhancing additives. Most suppliers of *Taq* and other thermostable DNA polymerases provide a unique optimized $MgCl_2$-free buffer with $MgCl_2$ in a separate vial for user titration. The second stage (steps 8 to 13) compares methods for preventing pre-PCR low-stringency primer extension, which can generate nonspecific products. This has come to be known as "hot start," whether one omits an essential reaction component prior to the first denaturing-temperature step or adds a reversible inhibitor of polymerase. Hot-start methods can greatly improve specificity, sensitivity, and yield. Use of any one of the hot-start approaches is strongly recommended if primer-dimers or other nonspecific products are generated or if relatively rare template DNA is contained in a complex mixture, such as viral nucleic acids in cell or tissue preparations. This protocol suggests some relatively inexpensive methods to achieve hot start, and lists several commercial hot-start options which may be more convenient, but of course more expensive.

Materials

Sterile H_2O
15 mM (L), 30 mM (M), and 45 mM (H) $MgCl_2$
10× $MgCl_2$-free PCR amplification buffer (see recipe)
25 mM 4dNTP mix (see recipe)
50 µM oligonucleotide primer 1: 50 pmol/µl in sterile H_2O (store at –20°C)
50 µM oligonucleotide primer 2: 50 pmol/µl in sterile H_2O (store at –20°C)
Template DNA: 1 µg mammalian genomic DNA or 1.0 to 100.0 pg of plasmid DNA
5 U/µl *Taq* DNA polymerase (native or recombinant; many suppliers)
Enhancer agents (optional; see recipe)
TaqStart Antibody (Clontech)
Mineral oil
Ficoll 400 (optional)
Tartrazine dye (optional)

Thin-walled PCR tubes
Automated thermal cycler

Contributed by Martha F. Kramer and Donald M. Coen
Current Protocols in Molecular Biology (1999) 15.1.1-15.1.15

Table 15.1.1 Master Mixes for Optimizing Reaction Components

Components	Final concentration	Per reaction	Master mix[a] (µl)			
			I	II	III	IV
10× PCR buffer	1×	10 µl	40.0	40.0	40.0	40.0
Primer 1	0.5 µM	1 µl	4.0	4.0	4.0	4.0
Primer 2	0.5 µM	1 µl	4.0	4.0	4.0	4.0
Template DNA	Undiluted	1 vol[b]	4 vol[b]	4 vol[b]	4 vol[b]	4 vol[b]
25 mM 4dNTP mix[c]	0.2 mM	0.8 µl	3.2	3.2	3.2	3.2
Taq polymerase	2.5 U	0.5 µl	2.0	2.0	2.0	2.0
DMSO[d] (20×)	5%	5 µl	—	20.0	—	—
Glycerol[d] (10×)	10%	10 µl	—	—	40.0	—
PMPE[d] (100×)	1%	1 µl	—	—	—	4.0
H_2O	—	To 90 µl	To 360	To 360	To 360	To 360

[a]Total volume = 360 µl (enough for $n + 1$ reactions).

[b]Template DNA volume ("vol") is generally 1 to 10 µl.

[c]If 2 mM 4dNTP mix is preferred, use 10 µl per reaction, or 40 µl for each master mix; adjust the volume of water accordingly.

[d]Substitute with other enhancer agents (see recipe in Reagents and Solutions) as available.

Additional reagents and equipment for DNA preparation (*UNITS 2.1-2.4*), agarose gel electrophoresis (*UNIT 2.5*), nondenaturing PAGE (*UNIT 2.7*), or sieving agarose gel electrophoresis (*UNIT 2.8*), restriction endonuclease digestion (*UNIT 3.1*), and Southern blotting and hybridization (*UNITS 2.9 & 6.4*)

NOTE: Do not use DEPC to treat water, reagents, or glassware.

NOTE: Reagents should be prepared in sterile, disposable labware, taken directly from its packaging, or in glassware that has been soaked in 10% bleach, thoroughly rinsed in tap water followed by distilled water, and if available, exposed to UV irradiation for ~10 min. Multiple small volumes of each reagent should be stored in screw-cap tubes. This will then serve as the user's own optimization "kit." Thin-walled PCR tubes are recommended.

Optimize reaction components

1. Prepare four reaction master mixes according to the recipes given in Table 15.1.1.

   Enhancing agents probably work by different mechanisms, such as protecting enzyme activity and decreasing nonspecific primer binding. However, their effects cannot be readily predicted—what improves amplification efficiency for one primer pair may decrease the amplification efficiency for another. Thus it is best to check a panel of enhancers during development of a new assay.

2. Aliquot 90 µl master mix I into each of three 0.5-ml thin-walled PCR tubes labeled I-L, I-M, and I-H. Similarly, aliquot mixes II through IV into appropriately labeled tubes. Add 10 µl of 15 mM $MgCl_2$ into one tube of each master mix (labeled L; 1.5 mM final). Similarly, aliquot 10 µl of 30 mM and 45 mM $MgCl_2$ to separate tubes of each master mix (labeled M and H, respectively; 3.0 and 4.5 mM final concentrations respectively).

It is helpful to set the tubes up in a three-by-four array to simplify aliquoting. Each of the three Mg^{2+} concentrations is combined with each of the four master mixes.

3. Overlay the reaction mixture with 50 to 100 µl mineral oil (2 to 3 drops).

To include hot start in the first step, overlay reaction mixes with oil before adding the $MgCl_2$, heat the samples to 95°C in the thermal cycler or other heating block, and add the $MgCl_2$ once the elevated temperature is reached. Once the $MgCl_2$ has been added, do not allow the samples to cool below the optimum annealing temperature prior to performing PCR.

Choose cycling parameters

4. Using the following guidelines, program the automated thermal cycler according to the manufacturers' instructions.

30 cycles:	30 sec	94°C	(denaturation)
	30 sec	55° (GC content ≤50%) *or* 60°C (GC content >50%)	(annealing)
	~60 sec/kb product sequence	72°C	(extension)

Cycling parameters are dependent upon the sequence and length of the template DNA, the sequence and percent complementarity of the primers, and the ramp times of the thermal cycler used. Thoughtful primer design will reduce potential problems (see Commentary). Denaturation, annealing, and extension are each quite rapid at the optimal temperatures. The time it takes to achieve the desired temperature inside the reaction tube, i.e., the ramp time, is usually longer than either denaturation or primer annealing. Thus, ramp time is a crucial cycling parameter. Manufacturers of the various thermal cyclers on the market provide ramp time specifications for their instruments. Ramp times are lower with thin-walled reaction tubes. The optimal extension time also depends on the length of the target sequence. Allow ~1 min/kb for this step for target sequences >1 kb, and as little as a 2-sec pause for targets <100 bases in length.

The number of cycles depends on both the efficiency of the reaction and the amount of template DNA in the reaction. Starting with as little as 100 ng of mammalian genomic DNA (~10^4 cell equivalents), after 30 cycles, 10% of the reaction should produce a band that is readily visible on an ethidium bromide–stained gel as a single predominant band. With more template, fewer cycles may suffice. With much less template, further optimization is recommended rather than increasing the cycle number. Greater cycle numbers (e.g., >40) can reduce the polymerase specific activity, increase nonspecific amplification, and deplete substrate (nucleotides). Many investigators lengthen the time for the last extension step—to 7 min, for example—to try to ensure that all the PCR products are full length.

These guidelines are appropriate for most commercially available thermal cyclers. For rapid cyclers, consult the manufacturers' protocols.

Analyze the product

5. Electrophorese 10 µl from each reaction on an agarose, nondenaturing polyacrylamide, or sieving agarose gel appropriate for the PCR product size expected. Stain with ethidium bromide.

For resolution of PCR products between 100 and 1000 bp, an alternative to nondenaturing polyacrylamide gels or sieving agarose is a composite 3% (w/v) NuSieve (FMC Bioproducts) agarose/1% (w/v) SeaKem (FMC Bioproducts) agarose gel. SeaKem increases the mechanical strength of the gel without decreasing resolution.

An alternative to ethidium bromide, SYBR Gold Nucleic Acid Gel Stain (Molecular Probes), is 25 to 100 times more sensitive than ethidium bromide, is more convenient to use, and permits optimization of 10- to 100-fold lower starting template copy number.

6. Examine the stained gel to determine which condition resulted in the greatest amount of product.

Table 15.1.2 Master Mixes for Optimizing First-Cycle Reactions

Components	Final concentration	Master mix (µl)			
		A	B	C	D
10× PCR buffer	1×	10	10	10	10
$MgCl_2$ (L, M, or H)	Optimal	10	10	10	10
Primer 1	0.5 µM	1.0	1.0	1.0	1.0
Primer 2	0.5 µM	1.0	1.0	1.0	1.0
Additive	Optimal	V[a]	V[a]	V[a]	V[a]
Template DNA	—[b]	V[a]	V[a]	V[a]	V[a]
25 mM 4dNTP mix[b]	0.2 mM	0.8	0.8	0.8	0.8
Taq polymerase	2.5 U	0.5	0.5	—	—
Taq pol + TaqStart	2.5 U	—	—	—	1.0
H_2O	To 100 µl	V[a]	V[a]	V[a]	V[a]
Preparation temperature		Room temperature	Ice slurry	Room temperature	Room temperature

[a]V, variable amount (total volume should be 100 µl).

[b]Use undiluted or diluted template DNA based on results obtained in step 6.

Minor, nonspecific products may be present even under optimal conditions.

7. To ensure that the major product is the correct one, digest an aliquot of the reaction with a restriction endonuclease known to cut within the PCR product. Check buffer compatibility for the restriction endonuclease of choice. If necessary, add Na^+ or precipitate in ethanol (*UNIT 2.1A*), then resuspend in the appropriate buffer. Electrophorese the digestion product on a gel to verify that the resulting fragments have the expected sizes.

Alternatively, transfer the PCR products to a nitrocellulose or nylon filter and hybridize with an oligonucleotide derived from the sequence internal to the primers. With appropriately stringent hybridization and washing conditions, only the correct product (and possibly some minor related products) should hybridize.

Optimize the first cycle

These optional steps optimize initial hybridization and may improve efficiency and yield. They are used when primer-dimers and other nonspecific products are detected, when there is only a very small amount of starting template, or when a rare sequence is to be amplified from a complex mixture. For an optimal reaction, polymerization during the initial denaturation and annealing steps should be prevented. Taq DNA polymerase activity can be inhibited by temperature (reaction B), physical separation (reaction C), or reversible antibody binding (reaction D). PCR without hot start is performed for comparison (reaction A).

8. Prepare four reaction mixtures using the optimal $MgCl_2$ concentration and additive requirement determined in step 6. Prepare the mixes according to the recipes in Table 15.1.2. Use the following variations for addition of *Taq* polymerase.

   a. Prepare reactions A and C at room temperature.

   b. Chill all components of reaction B in an ice slurry before they are combined.

   c. For reaction D, combine 1.0 µl TaqStart antibody with 4.0 µl of the dilution buffer provided with the antibody, add 1.0 µl *Taq* DNA polymerase (for 1:4:1 mixture of

these components) mix, and incubate 5 to 10 min at room temperature before adding to reaction mixture D (glycerol and PMPE are compatible with TaqStart antibody but DMSO will interfere with antibody binding).

To ensure that the reaction does not plateau and thereby obfuscate the results, use the smallest amount of template DNA necessary for visualization of the PCR product by ethidium bromide staining. Use the results from step 6 to decide how much template to use. If the desired product stains intensely, dilute the starting material as much as 1/100. If only a faint signal is apparent, use undiluted sample.

9. Overlay each reaction mixture with 50 to 100 µl mineral oil.

10. Heat all reactions 5 min at 94°C.

    It is most convenient to use the automated thermal cycler for this step and then initiate the cycling program directly.

11. Cool the reactions to the appropriate annealing temperature as determined in step 4. Add 0.5 µl *Taq* DNA polymerase to reaction C, making sure the pipet tip is inserted through the layer of mineral oil into the reaction mix.

    Time is also an important factor in this step. If the temperature drops below the annealing temperature and is allowed to remain low, nonspecific annealing will occur. Taq DNA polymerase retains some activity even at room temperature.

12. Begin amplification of all four reactions at once, using the same cycling parameters as before.

13. Analyze the PCR products on an agarose gel and evaluate the results as in steps 5 and 6.

14. Prepare a batch of the optimized reaction mixture, but omit *Taq* DNA polymerase, TaqStart antibody, PMPE, and 4dNTP mix—these ingredients should be added fresh just prior to use. If desired, add Ficoll 400 to a final concentration of 0.5% to 1% (v/v) and tartrazine to a final concentration of 1 mM.

    Adding Ficoll 400 and tartrazine dye to the reaction mix precludes the need for a gel loading buffer and permits direct application of PCR products to agarose or acrylamide gels. At these concentrations, Ficoll 400 and tartrazine do not decrease PCR efficiency and do not interfere with PMPE or TaqStart antibodies. Other dyes, such as bromphenol blue and xylene cyanol, do inhibit PCR. Tartrazine is a yellow dye and is not as easily visualized as other dyes; this may make gel loading more difficult.

    Ficoll 400 and tartrazine dye may be prepared as 10× stocks and stored indefinitely at room temperature.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see APPENDIX 2; for suppliers, see APPENDIX 4.

Enhancer agents

For a discussion of how to select enhancer agents, see Commentary.

5× stocks:
25% acetamide (20 µl/reaction; 5% final)
5 M *N,N,N*-trimethylglycine (betaine; 20 µl/reaction; 1 M final)
40% polyethylene glycol (PEG) 8000 (20 µl/reaction; 8% final)

10× stocks:
Glycerol (concentrated; 10 µl/reaction; 10% final)

20× stocks:
Dimethylsulfoxide (DMSO; concentrated 5 µl/reaction; 5% final)
Formamide (concentrated; 5 µl/reaction; 5% final)

100× stocks:
1 U/µl Perfect Match Polymerase Enhancer [Strategene; 1 µl (1 U) per reaction, final]
10 mg/ml acetylated bovine serum albumin (BSA) or gelatin (1 µl/reaction; 10 µg/ml final)
1 to 5 U/µl thermostable pyrophosphatase [PPase; Boehringer Mannheim; 1 µl (1 to 5 U) per reaction, final]
5 M tetramethylammonium chloride (TMAC; betaine hydrochloride; 1 µl/reaction; 50 mM final)
0.5 mg/ml *E. coli* single-stranded DNA-binding protein (SSB; Sigma; 1 µl/reaction; 5 µg/ml final)
0.5 mg/ml Gene 32 protein (Amersham Pharmacia Biotech; 1 µl/reaction; 5 µg/ml final)
10% Tween 20, Triton X-100, or Nonidet P-40 (1 µl/reaction; 0.1% final)
1 M $(NH_4)_2SO_4$ (1 µl/reaction; 10 mM final; use with thermostable DNA polymerases other than *Taq*)

$MgCl_2$-free PCR amplification buffer, 10×

500 mM KCl
100 mM Tris·Cl, pH 9.0 (at 25°C)
0.1% Triton X-100
Store indefinitely at –20°C

This buffer can be obtained from Promega; it is supplied with Taq DNA polymerase.

4dNTP mix

For 2 mM 4dNTP mix: Prepare 2 mM each dNTP in TE buffer, pH 7.5 (*APPENDIX 2*). Store up to 1 year at –20°C in 1-ml aliquots.

For 25 mM 4dNTP mix: Combine equal volumes of 100 mM dNTPs (Promega). Store indefinitely at –20°C in 1-ml aliquots.

Background Information

The theoretical basis of the polymerase chain reaction (PCR; see chapter introduction) was probably first described in a paper by Kleppe et al. (1971). However, this technique did not excite general interest until the mid-1980s, when Kary Mullis and co-workers at Cetus developed PCR into a technique that could be used to generate large amounts of single-copy genes from genomic DNA (Saiki et al., 1985, 1986; Mullis et al., 1986; Embury et al., 1987).

The initial procedure entailed adding a fresh aliquot of the Klenow fragment of *E. coli* DNA polymerase I during each cycle because this enzyme was inactivated during the subsequent denaturation step. The introduction of thermostable *Taq* DNA polymerase from *Thermus aquaticus* (Saiki et al., 1988) alleviated this tedium and facilitated automation of the thermal cycling portion of the procedure. *Taq* DNA polymerase also permitted the use of higher temperatures for annealing and extension, which improved the stringency of primer–template hybridization and thus the specificity of the products. This also served to increase the yield of the desired product.

Some applications for PCR are presented in detail in the following units; others are mentioned in the introduction to this chapter. All depend upon an optimized PCR. The basic protocol in this unit optimizes PCR for several variables, including $MgCl_2$ concentration, enhancing additives—dimethyl sulfoxide (DMSO), glycerol, or Perfect Match Polymerase Enchancer (PMPE)—and prevention of pre-PCR mispriming. These and other parameters can be extremely important, as every element of PCR can affect the outcome; see Critical Parameters and Troubleshooting for discussion of individual parameters.

There are several PCR optimization kits and proprietary enhancers on the market (Table 15.1.3). Optimization kits generally provide a panel of buffers in which the pH, buffer, nonionic detergents, and addition of $(NH_4)_2SO_4$ are varied, $MgCl_2$ may be added at several concentrations, and enhancers (e.g., DMSO, glycerol, formamide, betaine, and/or proprietary compounds) may be chosen. The protocol presented here is aimed at keeping the costs low and the options broad.

Critical Parameters and Troubleshooting

$MgCl_2$ concentration

Determining the optimum $MgCl_2$ concentration, which can vary even for different primers from the same region of a given template (Saiki, 1989), can have an enormous influence on PCR success. In this protocol three test concentrations are suggested—1.5 mM (L), 3.0 mM (M), and 4.5 mM (H). If further optimization is necessary, the $MgCl_2$ range can be extended or narrowed around the most successful concentration.

A 10× buffer optimized for a given enzyme and a separate vial of $MgCl_2$ are typically provided with the polymerase, so that the user may titrate the $MgCl_2$ concentration for their unique primer-template set. Note that some enhancers may broaden the $MgCl_2$ optimal range.

Reagent purity

For applications that amplify rare templates, reagent purity is the most important parameter, and avoiding contamination at every step is critical. This issue is addressed thoroughly in UNIT 15.3.

To maintain purity, store multiple small volumes of each reagent in screw-cap tubes.

For many applications, simply using high-quality reagents and avoiding nuclease contamination is sufficient. However, avoid one common reagent used to inactivate nucleases—diethylpyrocarbonate (DEPC). Even the tiny amounts of chemical left after treatment of water and autoclaving are enough to ruin a PCR.

Primer selection

This is the factor that is least predictable and most difficult to troubleshoot. Simply put, some primers just do not work. To maximize the probability that a given primer pair will work, pay attention to the following parameters.

General considerations. An optimal primer set should hybridize efficiently to the sequence of interest with negligible hybridization to other sequences present in the sample. If there are reasonable amounts of template available, hybridization specificity can be tested by performing oligonucleotide hybridization as in UNIT 6.4. The distance between the primers is rather flexible, ranging up to 10 kb. There is, however, a considerable drop-off in synthesis

Table 15.1.3 PCR Optimization Products

Optimization goal	Supplier	Product
Optimization support	Perkin-Elmer	Technical information in appendix to catalog
Optimization support	Promega	PCR troubleshooting program on the Internet: *http://www.promega.com/amplification/assistant*
Optimization kits	Boehringer-Mannheim, Invitrogen, Stratagene, Sigma, Epicentre Technologies, Life Technologies	Several buffers, Mg^{2+}, and enhancers which may include DMSO, glycerol, formamide, $(NH_4)_2SO_4$, and other unspecified or proprietary agents
Quick startup	Amersham Pharmacia Biotech	Ready-To-Go Beads "optimized for standard PCR" and Ready-To-Go RAPD Analysis Beads (buffer, nucleotides, *Taq* DNA polymerase)
Quick startup	Fisher	EasyStart PCR Mix-in-a-Tube—tubes prepackaged with wax beads containing buffer, $MgCl_2$, nucleotides, *Taq* DNA polymerase
Quick startup	Life Technologies	PCR SuperMix—1.1× conc.—premix containing buffer, $MgCl_2$, nucleotides, *Taq* DNA polymerase
Quick startup	Marsh Biomedical	Advanced Biochemicals Red Hot DNA Polymerase—a new rival for *Taq* polymerase with convenience features
Hot-start/physical barrier	Fisher, Life Technologies	Molecular Bio-Products HotStart Storage and Reaction Tubes—preadhered wax bead in each tube; requires manual addition of one component at high temperature
Hot-start/separate $MgCl_2$	Invitrogen	HotWax Mg^{2+} beads—wax beads contain preformulated $MgCl_2$ which is released at first elevated-temperature step
Hot-start/separate $MgCl_2$	Stratagene	StrataSphere Magnesium Wax Beads—wax beads containing preformulated Mg^{2+}
Hot Start/separate polymerase	Promega	TaqBead Hot Start Polymerase—wax beads encapsulating *Taq* DNA polymerase which is released at first elevated-temperature step
Hot-start/reversible inactivation of polymerase by antibody binding	Clontech	TaqStart Antibody, TthStart Antibody—reversibly inactivate *Taq* and *Tth* DNA polymerases until first denaturation at 95°C
Hot-start/antibody binding	Life Technologies	PlatinumTaq—contains PlatinumTaq antibody
Hot-start/antibody binding	Sigma	JumpStart Taq—contains TaqStart antibody
Hot-start/reversible chemical modification	Perkin-Elmer	AmpliTaq Gold—activated at high temperature
Hot-start/reversible chemical modification	Qiagen	HotStarTaq DNA Polymerase—activated at high temperature
Enhancer	Boehringer Mannheim, New England Biolabs	*Tth* pyrophosphatase, thermostable
Enhancer	Clontech	GC-Melt (in Advantage-GC Kits)—proprietary
Enhancer	CPG	Taq-FORCE Amplification System and MIGHTY Buffer—proprietary
Enhancer	Fisher	Eppendorf MasterTaq Kit with TaqMaster Enhancer—proprietary
Enhancer	Life Technologies	PCRx Enhancer System—proprietary
Enhancer	Promega	*E.coli* Single Stranded Binding Protein (SSB)
Enhancer	Qiagen	Q-Solution—proprietary
Enhancer	Stratagene	Perfect Match Polymerase Enhancer—proprietary
Enhancer	Stratagene	TaqExtender PCR Additive—proprietary

efficiency with distances >3 [redacted] Jeffreys et al., 1988). (For amplification of very long products, see *UNIT 15.6*). Small distances between primers, however, lessen the ability to obtain much sequence information or to reamplify with nested internal oligonucleotides, should that be necessary.

Design primers to allow demonstration of the specificity of the PCR product. Be sure that there are diagnostic restriction endonuclease sites between the primers or that an oligonucleotide can detect the PCR product specifically by hybridization.

Several computer programs can assist in primer design. These are most useful for avoiding primer sets with intra- and intermolecular complementarity, which can dramatically raise the effective T_m. Given the abundance of primers relative to template, this can preclude template priming. Computer primer design is not foolproof. If possible, start with a primer or primer set known to efficiently prime extensions. In addition, manufacturers' Web sites offer technical help with primer design.

Complementarity to template. For many applications, primers are designed to be exactly complementary to the template. For others, however, such as engineering of mutations or new restriction endonuclease sites (*UNIT 8.5*), or for efforts to clone or detect gene homologs where sequence information is lacking, base-pair mismatches will be intentionally or unavoidably created. It is best to have mismatches (e.g., in a restriction endonuclease linker) at the 5′ end of the primer. The closer a mismatch is to the 3′ end of the primer, the more likely it is to prevent extension. If cloned template is available, primers can be checked for suitability by using them in a sequencing reaction with *Taq* DNA polymerase (*UNIT 7.4*).

The use of degenerate oligonucleotide primers to clone genes where only protein sequence is available, or to fish out gene homologs in other species, has sometimes been successful—but it has also failed an untold (and unpublished) number of times. When the reaction works it can be extremely valuable, but it can also generate seemingly specific products that require much labor to identify and yield no useful information. The less degenerate the oligonucleotides, especially at the 3′ end, the better. Caveat emptor.

Primer length. A primer should be 20 to 30 bases in length. It is unlikely that longer primers will help increase specificity significantly.

Primer sequence. Design [redacted]mers with a GC content similar to that of the template. Avoid primers with unusual sequence distributions, such as stretches of polypurines or polypyrimidines as their secondary structure can be disastrous. It is worthwhile to check for potential secondary structure using one of the appropriate computer programs that are available.

"Primer-dimers." Primer-dimers are a common artifact most frequently observed when small amounts of template are taken through many amplification cycles. They form when the 3′ end of one primer anneals to the 3′ end of the other primer, and polymerase then extends each primer to the end of the other. The ensuing product can compete very effectively against the PCR product of interest. Primer-dimers can best be avoided by using primers without complementarity, especially in their 3′ ends. Should they occur, optimizing the $MgCl_2$ concentration may minimize their abundance relative to that of the product of interest.

Template

Aside from standard methods for preparing DNA (*UNITS 2.1-2.4*), a number of simple and rapid procedures have been developed for particular tissues (Higuchi, 1989). Even relatively degraded DNA preparations can serve as useful templates for generation of moderate-sized PCR products. The two main concerns regarding template are purity and amount.

A number of contaminants found in DNA preparations can decrease the efficiency of PCR. These include urea, the detergent SDS (whose inhibitory action can be reversed by nonionic detergents), sodium acetate, and, sometimes, components carried over in purifying DNA from agarose gels (Gelfand, 1989; Gyllensten, 1989; K. Hicks and D. Coen, unpub. observ.). Additional organic extractions, ethanol precipitation from 2.5 M ammonium acetate, and/or gel purification on polyacrylamide rather than agarose, can all be beneficial in minimizing such contamination if the simplest method (precipitating the sample with ethanol and repeatedly washing the pellet with 70% ethanol) is not sufficient.

Clearly the amount of template must be sufficient to be able to visualize PCR products using ethidium bromide. Usually 100 ng of genomic DNA is sufficient to detect a PCR product from a single-copy mammalian gene. Using too much template is not advisable when optimizing for $MgCl_2$ or other parameters, as

it may obscure differences in amplification efficiency. Moreover, too much template may decrease efficiency due to contaminants in the DNA preparation.

Amount of template, especially in terms of the amount of target sequence versus nonspecific sequences, can have a major effect on the yield of nonspecific products. With less target sequence, it is more likely that nonspecific products will be seen. For some applications, such as certain DNA sequencing protocols (*UNIT 15.2*) where it is important to have a single product, gel purification of the specific PCR product and reamplification are advisable.

Taq and other thermostable DNA polymerases

Among the advantages conferred by the thermostability of *Taq* DNA polymerase is its ability to withstand the repeated heating and cooling inherent in PCR and to synthesize DNA at high temperatures that melt out mismatched primers and regions of local secondary structure. The enzyme, however, is not infinitely resistant to heat, and for greatest efficiency it should not be put through unnecessary denaturation steps. Indeed, some protocols (e.g., *UNIT 15.3* and the "hot start" method described here) recommend adding it after the first denaturation step.

Increasing the amount of *Taq* DNA polymerase beyond 2.5 U/reaction can sometimes increase PCR efficiency, but only up to a point. Adding more enzyme can sometimes increase the yield of nonspecific PCR products at the expense of the product of interest. Moreover, *Taq* DNA polymerase is not inexpensive.

A very important property of *Taq* DNA polymerase is its error rate, which was initially estimated at 2×10^{-4} nucleotides/cycle (Saiki et al., 1988). The purified enzyme supplied by manufacturers lacks a proofreading 3′→5′ exonuclease activity, which lowers error rates of other polymerases such as the Klenow fragment of *E. coli* DNA polymerase I. For many applications, this does not present any difficulties. However, for sequencing clones derived from PCR, or when starting with very few templates, this can lead to major problems. Direct sequencing of PCR products (*UNIT 15.2*), sequencing numerous PCR-generated clones, and/or the use of appropriate negative controls can help overcome these problems. Alternatively, changing reaction conditions (Eckert and Kunkel, 1990) or changing to a non–*Taq* DNA polymerase (with greater fidelity) may be useful.

Another important property of *Taq* DNA polymerase is its propensity for adding nontemplated nucleotides to the 3′ ends of DNA chains. This can be especially problematic in cloning PCR products. It is frequently necessary to "polish" PCR products with enzymes such as other DNA polymerases before adding linkers or proceeding to blunt-end cloning. Conversely, addition of a nontemplated A by *Taq* DNA polymerase can be advantageous in cloning (see *UNIT 15.7*).

Table 15.1.4 lists currently available thermostable DNA polymerases by generic and trade names, the original source of native and recombinant enzymes, the supplier, the end generated (3′A addition versus blunt), and associated exonuclease activities. A 3′ to 5′ exonuclease activity is proofreading. Removal of the 5′ to 3′ exonuclease activity of *Taq* DNA polymerase (N-terminal deletion) is reported to produce a higher yield. A 5′ to 3′ exonuclease activity may degrade the primers somewhat. Proofreading enzymes synthesize DNA with higher fidelity and can generate longer products than *Taq*, but tend to generate low yields. Enzyme blends (Table 15.1.5) have been optimized for increased fidelity and length along with sensitivity and yield.

Hot start

What happens prior to thermal cycling is critical to the success of PCR. *Taq* DNA polymerase retains some activity even at room temperature. Therefore, under nonstringent annealing conditions, such as at room temperature, products can be generated from annealing of primers to target DNA at locations of low complementarity or having complementarity of just a few nucleotides at the 3′ ends. The latter would in effect create new templates "tagged" with the primer sequences. Subsequent cycles amplify these tagged sequences in abundance, both generating nonspecific products and possibly reducing amplification efficiency of specific products by competition for substrates or polymerase. Thus conditions preventing polymerization prior to the first temperature-controlled steps are desirable. In this protocol, three methods of inhibiting polymerization prior to the temperature-controlled step are compared. These include physical separation of an essential reaction component prior to the first denaturation step, cooling reagents to 0°C, and revers-

Table 15.1.4 Thermostable DNA Polymerases

DNA polymerase		Biological source	Supplier	Product ends	Exonuclease activity
Generic name	Trade name				
Pfu	—	*Pyrococcus furiosus*	Stratagene, Promega	Blunt	3′-5′ (proofreading)
Pfu (exo-)	—	*Pyrococcus furiosus*	Stratagene	Blunt	No
Psp	Deep Vent	*Pyrococcus* sp.GB-D	New England Biolabs	Blunt	3′-5′ (proofreading)
Psp (exo-)	Deep Vent (exo-)	*Pyrococcus* sp.GB-D	New England Biolabs	Blunt	No
Pwo		Pyrococcus woesei	Boehringer Mannheim	Blunt	3′-5′ (proofreading)
Taq (native and/or recombinant)	—	*Thermus aquaticus*	Ambion, Amersham Pharmacia Biotech, Boehringer Mannheim, Clontech, Fisher, Life Technologies, Marsh Biomedical, Perkin Elmer, Promega, Qiagen, Sigma, Stratagene	3′A	5′-3′
Taq, N-terminal deletion	Stoffel fragment Klen-Taq	*Thermus aquaticus*	Perkin-Elmer, Sigma	3′A	No
Tbr	DyNAzyme	*Thermus brocianus*	MJ Research	—[a]	5′-3′
Tfl		*Thermus flavus*	Promega, Epicentre Technologies	Blunt	—[a]
Tli	Vent	*Thermococcus litoralis*	New England Biolabs (Vent), Promega	Blunt	3′-5′ (proofreading)
Tli (exo-)	Vent (exo-)	*Thermococcus litoralis*	New England Biolabs	Blunt	No
Tma	UlTma	*Thermotoga maritima*	Perkin-Elmer	Blunt	3′-5′ (proofreading)
Tth	—	*Thermus thermophilus*	Amersham Pharmacia Biotech, Boehringer Mannheim, Epicentre Technologies, Perkin Elmer, Promega	3′A	5′-3′

[a]No information at this time.

ibly blocking enzymatic activity with an antibody.

Denaturation of the template before *Taq* polymerase or $MgCl_2$ is added to the reaction provides a dramatic improvement in specificity and sensitivity in many cases (Chou et al., 1992). The main drawback of this method is that it requires opening the reaction tubes a second time to add the essential missing component. This creates both an inconvenience and an increase in the risk of contamination, an important consideration when testing for the presence of a given sequence in experimental or clinical samples.

Cooling all components of the reaction mixture to 0°C prior to mixing is more convenient and the least expensive method but is also the least reliable. Transferring the PCR reaction tubes from the ice slurry to a 95°C preheated thermocycler block may improve the chance of success.

Reversible inhibition of *Taq* DNA polymerase by TaqStart antibody (Clontech) is the most convenient and very effective (Kellogg et al., 1994). Complete reactions can be set up, overlaid with oil, and stored at 4°C for up to several hours prior to thermal cycling with no loss of sensitivity or specificity compared to the other hot start methods (M.F.K. and D.M.C.,

Table 15.1.5 Thermostable DNA Polymerase Blends

Product (trade name)	Supplier	Thermostable DNA polymerases and other components
Expand High Fidelity, Expand Long Template, and Expand 20kb PCR Systems	Boehringer Mannheim	*Taq* + *Pwo*
KlenTaq LA Polymerase Mix	Clontech, Sigma	KlenTaq-1 (5′-exonuclease deficient *Taq*) + unspecified proofreading polymerase
Advantage-HF PCR Kit	Clontech	KlenTaq-1 + unspecified proofreading polymerase + TaqStart Antibody
Advantage-cDNA and Advantage-GC cDNA Polymerase Mixes and Kits	Clontech	KlenTaq-1 + unspecified proofreading polymerase + TaqStart Antibody; GC Kit contains GC Melt
Advantage Genomic and Advantage-GC Genomic Polymerase Mixes and Kits	Clontech	*Tth* + unspecified proofreading polymerase + TthStart Antibody; GC Kit contains GC Melt
eLONGase Enzyme Mix	Life Technologies	*Taq* + *Psp* + unspecified proofreading polymerase(s) + eLONGase Buffer
Platinum Taq DNA Polymerase	Life Technologies	*Taq* + *Psp* + Platinum *Taq* Antibody
Platinum High Fidelity DNA Polymerase	Life Technologies	*Taq* + *Psp* + *Taq* Antibody
DyNAzyme EXT Polymerase	MJ Research	*Tbr* with unspecified enhancer
GeneAmp XL PCR and XL RNA PCR Kits	Perkin-Elmer	*Tth* + *Tli*
OmniBase Sequencing Enzyme Mix	Promega	Unspecified proofreading polymerase(s) with thermostable pyrophosphatase
AccuTaq LA DNA Polymerase Mix	Sigma	*Taq* + unspecified proofreading polymerase
TaqPlus Long and TaqPlus Precision PCR Systems	Stratagene	*Pfu* + *Taq*; TaqPlus Precision Reaction Buffer (proprietary)
Accurase Fidelity PCR Enzyme Mix; Calypso High Fidelity Single Tube RT-PCR System	Tetralink	*Thermus sp.* + *Thermococcus sp.*; Calypso also contains AMV-RT

unpub. observ.). Cycling is initiated immediately following 5-min denaturation of the antibody at 94°C. DMSO inhibits antibody binding and should not be used with TaqStart.

Several hot-start products are now commercially available (Table 15.1.3). Success with each may depend on strict adherence to the manufacturer's protocols, even on a specific thermocycler. Wax barrier and reversible antibody binding methods are more forgiving, while chemical modifications have more stringent activation temperature requirements.

Deoxyribonucleoside triphosphates

In an effort to increase efficiency of PCR, it may be tempting to increase the concentration of dNTPs. Don't. When each dNTP is 200 μM, there is enough to synthesize 12.5 μg of DNA when half the dNTPs are incorporated. dNTPs chelate magnesium and thereby change the effective optimal magnesium concentration. Moreover, dNTP concentrations >200 μM each increase the error rate of the polymerase. Millimolar concentrations of dNTPs actually inhibit *Taq* DNA polymerase (Gelfand, 1989).

The protocol in this unit calls for preparing 4dNTPs in 10 mM Tris·Cl/1 mM EDTA (TE buffer), pH 7.4 to 7.5. This is easier and less prone to disaster than neutralization with sodium hydroxide. However, EDTA also chelates magnesium, and this should be taken into account if stocks of dNTPs are changed. Alternatively, to lower the risk of contamination, a 4dNTP mix can be made by combining equal volumes of commercially prepared stocks.

Enhancers

Enhancers are used to increase yield and specificity and to overcome difficulties encountered with high GC content or long templates.

Nonionic detergents (Triton X-100, Tween 20, or Nonidet P-40) neutralize charges of ionic detergents often used in template preparation, and should be used in the basic reaction mixture, rather than as optional enhancers. Higher yields can be achieved by stabilizing/enhancing the polymerase activity with enzyme-stabilizing proteins (BSA or gelatin), enzyme-stabilizing solutes such as betaine or betaine·HCl (TMAC), enzyme-stabilizing solvents (glycerol), solubility-enhancing solvents (DMSO or acetamide), molecular crowding solvents (PEG), and polymerase salt preferences [$(NH_4)SO_4$ is recommended for polymerases other than *Taq*]. Greater specificity can be achieved by lowering the T_M of dsDNA (using formamide), destabilizing mismatched-primer annealing (using PMPE or hot-start strategies), and stabilizing ssDNA (using *E. coli* SSB or Gene 32 Protein). Amplification of high-GC-content templates can be improved by decreasing the base pair composition dependence of the T_M of dsDNA (with betaine; Rees et al., 1993). Betaine is an osmolyte widely distributed in plants and animals and is nontoxic, a feature that recommends it for convenience in handling, storage, and disposal. Betaine may be the proprietary ingredient in various commercial formulations. For long templates, a higher pH is recommended (pH 9.0). The pH of Tris buffer decreases at high temperatures, long-template PCR requires more time at high temperatures, and increased time at lower pH may cause some depurination of the template, resulting in reduced yield of specific product. Inorganic phosphate (PPi), a product of DNA synthesis, may accumulate with amplification of long products to levels that may favor reversal of polymerization. Accumulation of PPi may be prevented by addition of thermostable PPase. When large numbers of samples are being analyzed, the convenience of adding PCR products directly to a gel represents a significant time savings. Some companies combine their thermostable polymerase with a red dye and a high density component to facilitate loading of reaction products onto gels without further addition of loading buffer.

Thermal cycling parameters

Each step in the cycle requires a minimal amount of time to be effective, while too much time can be both wasteful and deleterious to the DNA polymerase. If the amount of time in each step can be reduced, so much the better.

Denaturation. It is critical that complete strand separation occur during the denaturation step. This is a unimolecular reaction which, in itself, is very fast. The suggested 30-sec denaturation used in the protocol ensures that the tube contents reach 94°C. If PCR is not working, it is well worth checking the temperature inside a control tube containing 100 µl water. If GC content is extremely high, higher denaturation temperatures may be necessary; however, *Taq* DNA polymerase activity falls off quickly at higher temperatures (Gelfand, 1989). To amplify a long sequence (>3 kb), minimize the denaturation time to protect the target DNA from possible effects, such as depurination, of lowered pH of the Tris buffer at elevated temperatures.

Annealing. It is critical that the primers anneal stably to the template. Primers with relatively low GC content (<50%) may require temperatures lower than 55°C for full annealing. On the other hand, this may also increase the quantity of nonspecific products. For primers with high GC content, higher annealing temperatures may be necessary. It can be worthwhile, although time-consuming, to experiment with this parameter. Two manufacturers have thermal cyclers on the market which are capable of forming a temperature gradient across the heating units, thus permitting annealing temperature optimization in one run. These are Stratagene's Robocyclers, and Eppendorf's Master Cycler. As with denaturation, the time for this step is based mainly on the time it takes to reach the proper temperature, because the primers are in such excess that the annealing reaction occurs very quickly.

Extension. The extension temperature of 72°C is close to the optimal temperature for *Taq* DNA polymerase (~75°C), yet prevents the primers from falling off. Indeed, primer extension begins during annealing, because *Taq* DNA polymerase is partially active at 55°C and even lower temperatures (Gelfand, 1989).

The duration of extension depends mainly on the length of the sequence to be amplified. A duration of 1 min per kb product length is usually sufficient.

Certain protocols, including others in this chapter, end the PCR with a long final extension time in an attempt to try to make products as complete as possible.

Ramp time. Ramp time refers to the time it takes to change from one temperature to another. Using water baths and moving samples

manually from temperature to temperature probably gives the shortest ramp times, which are mainly the time required for the tube's contents to change temperature. Different thermal cyclers have different ramp times; basically, the shorter the better.

The Stratagene Robocycler uses a robotic arm to move samples from one constant-temperature block to another, virtually eliminating ramp time. Rapid cyclers utilize positive-displacement pipet tips or capillary tubes for the PCR reactions, dramatically reducing the ramp times.

Generally, the more "high-performance" thermal cyclers with short ramp times are proportionally more costly. There are many new thermal cyclers on the market priced below $5000, which perform quite well (Beck, 1998).

Anticipated Results

Starting with ≥100 ng mammalian DNA (≥10^4 molecules), the basic protocol can be used to determine which $MgCl_2$ concentration, enhancing additive, and initial conditions will yield a predominant PCR product from a single-copy sequence that is readily visible on an ethidium bromide–stained gel. It is possible that other minor products will also be visible.

Time Considerations

The basic protocol can be completed in a single day. Assembly of the reaction mixtures should take ~1 hr. Cycling should take less than 3 hr. Preparing, running, and staining the gel should take another few hours. Further checks on specificity of the product such as restriction endonuclease digestion or Southern blot hybridization will take another few hours or days, respectively.

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Saiki, R.K., Gelfand, D.H., Stoffel, S., Scharf, S.J., Higuchi, R., Horn, G.T., Mullis, K.B., and Erlich, H.A. 1988. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science* 239:487-491.

Key Reference

Saiki et al., 1988. See above.

Demonstrates the ease and power of PCR using Taq DNA polymerase.

Internet Resources

http://www.perkin-elmer.com

http://www.promega.com

http://www.stratagene.com

Most companies can be accessed at "http://company-name.com". Many such company Web sites provide protocols, information on primer design, and other PCR assistance.

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UNIT 15.2

Direct DNA Sequencing of PCR Products

This unit describes two basic approaches for direct DNA sequencing of polymerase chain reaction (PCR) products. Either approach permits the rapid characterization of sequences of interest, without the need for library construction or screening.

PCR products can be sequenced using either the dideoxy (Sanger) approach or the chemical (Maxam-Gilbert) approach. Dideoxy sequencing methods are presented in the first basic and the first three alternate protocols. In the first basic and first alternate protocols, the target sequence is amplified and an excess of one strand of the target sequence (relative to its complement) is then generated by "asymmetric PCR," where one primer is present in vast excess over the other. This single-stranded product serves as the template for conventional dideoxy sequencing methods (*UNIT 7.4*). The second alternate protocol describes how PCR products can be prepared so that they will be suitable templates for double-stranded dideoxy sequencing methods (*UNIT 7.4*). The third alternate protocol uses λ exonuclease to generate single-stranded template from double-stranded PCR products.

Chemical sequencing methods are presented in the second basic protocol, where an end-labeled product is used, and in the fourth alternate protocol, where an unlabeled product is characterized using the genomic sequencing approach. The dideoxy method is somewhat simpler, and can be used for most applications, especially where short stretches of sequence are being characterized. The chemical sequencing method, although requiring more steps, is well suited for larger-scale sequencing projects where a number of separate sequences need to be amplified and determined, as in "multiplex sequencing" (Church and Kieffer-Higgins, 1988; see commentary).

BASIC PROTOCOL

GENERATING SINGLE-STRANDED PRODUCTS FOR DIDEOXY SEQUENCING BY ASYMMETRIC PCR

In this approach, an excess of one amplified strand (relative to its complement) is generated by the addition of one primer in vast excess over the other. The resulting excess of single-stranded product is then used as a template for the production of the dideoxy-terminated chains from which the sequence is derived.

Materials

Oligonucleotide primers 1 and 2
^{32}P-labeled dNTPs (optional; *UNIT 3.4*)
10 M ammonium acetate (*APPENDIX 2*)
100% and 70% ethanol, room temperature
0.1× TE buffer, pH 8.0 (*APPENDIX 2*)
Centricon 30 or 100 column (optional; Amicon)
Additional reagents and equipment for PCR (*UNIT 15.1*), electrophoresis using agarose gels (*UNIT 2.5*) or nondenaturing polyacrylamide gels (*UNIT 2.7*), Southern blotting and hybridization (*UNITS 2.9 & 6.4*), ethanol precipitation (*UNIT 2.1*), and dideoxy sequencing (*UNIT 7.4*)

1. Assemble a PCR with optimized components (*UNIT 15.1*) but use ~100:1 ratio of the two oligonucleotide primers.

   The ideal amounts of the primers should be determined empirically, but will generally range from 0.2 to 1 pmol for the limiting primer, and from 10 to 30 pmol for the primer present in excess. Because the primers are present in substantial excess under standard PCR conditions (~50 pmol of each primer), the limiting primer must be exhausted prior to the completion of PCR in order to produce an excess of one strand.